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Chapter 2

Clustering of Olive Pollens into Model Cultivars on the Basis of Their Allergenic Content

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Additional information is available at the end of the chapter

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1. Introduction

Olive pollen allergy is a leading cause of seasonal allergic disease in the Mediterranean countries, where olive trees are intensively cultivated and pollen grain count reaches very high levels during the pollination season (Wheeler, 1992, Liccardi et al., 1996). The level of sensitization to olive pollen among the general population is directly related to the abundance of trees as this determines allergen exposure. Nevertheless, apart from tree abundance, other factors such as genetic background may influence the incidence of sensitization to olive pollen even in areas of very high exposition (Geller-Bernstein et al. 1996).Olive trees have been cultivated in the Mediterranean basin for several millennia and this has led to the selection of a wide variety of cultivars with agronomic importance. Olive germplasm is exceptionally wide, with more than 250 cultivars only in Spain (Barranco and Rallo, 2005), probably as a direct consequence of intensive cultivation.

Material commonly used for clinical and biological analysis corresponds in most cases to commercially available pollen, obtained from uncertain varietal sources. Previous studies have determined that most allergens isolated and characterized up to date are highly polymorphic (Villalba et al. 1993, 1994; Lombardero et al. 1994; Asturias et al. 1997; Alché et al. 1998; Tejera et al. 1999; Huecas et al. 2001; Martínez et al. 2002; Jiménez-López et al. 2012). Besides polymorphism, olive cultivars display broad differences in the expression levels for many allergens (Carnés et al. 2002; Conde Hernandez et al. 2002; Castro et al. 2003; Morales 2012) as well as in the number and molecular characteristics of the expressed allergen isoforms (Hamman-Khalifa et al. 2003, 2008; Hamman-Khalifa 2005; Castro et al. 2010; Jiménez-López et al. 2012). These differences are in a certain degree

maintained over the years, and have been demonstrated to be associated to the genetic background of the different olive cultivars (Fernandez Caldas et al. 2007; Morales 2012; Morales et al. this volume). Differences in the allergen composition of the extracts, particularly as regard to the olive pollen major allergen Ole e 1, are responsible of large differences in the biological potency of the extracts. Thus, Castro et al. (2003) analysed the allergenicity and Ole e 1 content in pollen samples of 10 cultivars of olive trees, and compared it to a commercial extract with no indication on varietal origin, probably representing a mixture of several cultivars. The authors found that there are important differences in the content of this major allergen and that Ole e 1 abundance correlated with total allergenicity when extracts were tested by skin prick test (SPT) on allergic patients. Interestingly some patients (about 10%) did not react with a commercial extract and only reacted to extracts coming from specific cultivars. These findings may have important implications in both diagnosis and therapy of olive pollen allergy, and in the efficacy and safety of the preparations used for specific immunotherapy (SIT) (Castro et al., 2003; Alché et al. 2007; Hamman-Khalifa et al. 2008; Jiménez-López 2008; Morales 2012).

The basis for personalized SIT, based in the individual usage of olive cultivar extracts, have been described and are protected by several Spanish patents (Alché et al. 2005, 2006). However, and as handling and characterization of a large number of cultivar extracts is impracticable under industrial and clinical standards, the present work intends to define a limited number of model cultivar, characterized by distinctive pollen allergen profiles. For this purpose, a number of olive pollen extracts have been analysed in their content for several relevant allergens. After appropriate quantitation, several model cultivars have been defined to group the cultivars analysed. This model can be used as the basis for a future classification and inclusion of the numerous olive cultivars available.

2. Materials and methods

2.1. Pollen samples

Olea europaea L. pollen samples were obtained during May and June of 2005-2010 from cultivated trees of the following cultivars: 'Picual', 'Manzanilla', 'Arbequina', 'Blanqueta', 'Cornicabra', 'Verdial', 'Lechín', 'Hojiblanca', 'Lucio'and 'Loaime'. Pollen samples were collected from numerous branches of at least two trees of each cultivar by shaking flowering shoots inside paper bags. Prior to its storage in liquid nitrogen, the harvested pollen was sieved through a 150 μm mesh in order to eliminate fallen corollas, anthers and other rests. After light microscopy observation, foreign-species pollen was estimated to be <0.1% and other plant parts <0.5% for all the cultivars used.

2.2. Preparation of crude protein extracts and SDS-PAGE

Crude protein extracts were obtained by stirring 1 g of pollen for each cultivar in 10 ml extraction buffer (0.01 M ammonium bicarbonate, pH 8.0, and 2 mM phenylmethylsulfonyl

fluoride) for 8 h at 4°C. After centrifugation (2 x 30 minutes at 14,000 rpm at 4°C), the supernatants were filtered through a 0.2 μm filter, and stored in aliquots at –20°C. Protein concentration in the different samples was measured using the Bio-Rad reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as standard.

Proteins (30 μg per lane) and Mw1 (New England BioLabs, Ipswich, MA, USA) and Mw2 standards (MBI Fermentas, Vilnius, Lithuania) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% gels in a MiniProtean II system (Bio-Rad). The resulting gels were stained with Coomassie blue. The same procedure described here was applied to a commercially available extract used for olive pollen allergy diagnosis.

2.3. Immunoblotting

Gels obtained as described above were transferred onto BioTrace® polyvinylidene difluoride (PVDF) membranes (Pall BioSupport, Port Washington, NY, USA) at 100 V for 1.5 hours using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Immunoblots were performed independently in the case of Ole e 1 (Figure 2) and Ole e 2 (Figure 3). Ole e 5 and Ole e 9 were simultaneously detected in the same membrane (Figure 4). Prior to the treatment with antibodies, the membranes were blocked with TBST buffer (Tris buffered saline: TBS + 0.3% v/v Tween 20) + 10% w/v dried skimmed milk.

The membranes were probed with antibodies to the following allergenic proteins: Ole e 1, (olive pollen major allergen), Ole e 2 (profilin), Ole e 5 (Cu,Zn superoxide dismutase) and Ole e 9 (1,3-β-glucanase). The anti-Ole e 1 mAb was kindly provided by Dr. Carlos Lahoz (Fundación Jiménez Díaz, Madrid, Spain) (Lauzurica et al. 1988). The anti-Ole e 2 polyclonal antibody (PoAb) was produced by immunization of rabbits with a keyhole limpet hemocyanin (KLH)-linked synthetic peptide (AQSATFPQFKPEEM) designed from the predicted amino acid sequence of an olive profilin (Ole e 2). Specificity of the antibody was already reported by Western blotting experiments and immunolocalization of the allergen (Morales et al. 2008). The anti-Ole e 9 polyclonal Ab was produced as described above using a synthetic peptide (YPYFAYKNQPTPDT) from the Ole e 9 amino acid sequence (Huecas et al. 2001; Duffort et al. 2006). Finally, we also purchased a commercially available PoAb that recognizes a chloroplastidic isoform of Cu/Zn-superoxide dismutase (SOD) from *Arabidopsis thaliana* (Agrisera, city, Sweden, Product No AS06 170), with probed cross-reactivity to Ole e 5 (Zafra 2007).

Primary Abs were diluted in blocking solution and incubated for 2 h at room temperature, whereas secondary Abs were diluted in TBST buffer and incubated for 1 h at room temperature in the dark. After Ab incubation, membranes were rinsed in TBST buffer four times for 5 min each. The different Abs used in this work and their corresponding dilutions are summarized in Table 1. Each experiment described below was repeated in triplicate. Negative controls included preimmune serum in the cases of Ole e 2 and Ole e 9.

Target	Primary antibody	Dilution	Secondary antibody	Dilution
Ole e 1	Mouse anti-olive Ole e 1 mAb (Lauzurica et al. 1988)	1:20,000	Goat anti-mouse IgG Ab, Alexa fluor 488-conjugated (Molecular Probes)	1:10,000
Ole e 2	Rabbit anti-olive Ole e 2 PoAb (Morales et al. 2008)	1:20,000	Donkey anti-rabbit IgG (Fab fragment) Ab, Cy3-conjugated (Jackson ImmunoResearch)	1:10,000
Ole e 5	Rabbit anti-Cu/Zn SOD PoAb (Agrisera Prod. No. AS06 170)	1:250	Goat anti-rabbit IgG Ab, Alexa fluor 633-conjugated (Molecular Probes)	1:10,000
Ole e 9 (N- domain)	Rabbit anti-olive Ole e 9 PoAb	1:10,000	Goat anti-rabbit IgG Ab, Alexa fluor 633-conjugated (Molecular Probes)	1:10,000

Table 1. Antibodies and dilutions used for immunoblotting experiments. mAb: monoclonal antibody; PoAb: polyclonal antibody.

Imaging was carried out with a Pharos FX Plus Molecular Imager (Bio-Rad) using the Quantity One v4.6.2 software (Bio-Rad).

2.4. Absolute and relative quantitation of allergens

The intensity of each fluorescent band was calculated using the quantitation tools of the Quantity One v4.6.2 software. In order to increase sensitivity of measurements and to avoid disturbing factors like the intensity of the background, the presence of individual non-specific spots, etc., two different methods for quantitation were used:

- For each allergen studied, reactive bands were identified, their optical density individually measured and then their absolute values added for each cultivar. Relative percentages of each allergen were then calculated for each cultivar, taking the cultivar with the highest optical density as the reference, which was assigned 100%.
- Simultaneous measurement of the optical density corresponding to all reactive bands from a given allergen in each cultivar was also performed. As before, relative percentages were also calculated, referred to the cultivar with the highest optical density, which was assigned 100%.

Finally, average of the percentages calculated by both methods was worked out, and the resulting percentages were newly made relative to the cultivar with the highest percentage, which was re-assigned 100%.

3. Results

3.1. SDS-PAGE protein profiles

Figure 1 shows the protein profiles of the extracts analysed after SDS-PAGE and Coomassie staining. The patterns observed for the major protein species were somewhat similar for all the cultivars tested. However, clear quantitative differences were distinguished, from which the most conspicuous were those in the protein range of 17-20 kDa. Proteins within this range were relatively abundant in the extracts corresponding to the cvs. 'Picual', 'Manzanilla', 'Cornicabra', 'Hojiblanca', 'Loaime', 'Blanqueta' and 'Lucio'.

When the commercial pollen extract was assayed by SDS-PAGE, a protein profile similar to the profile corresponding to the individual cultivars was observed, although several bands were absent or poorly resolved. Proteins in the range 17-20 kDa represented a low proportion of the total protein for this extract.

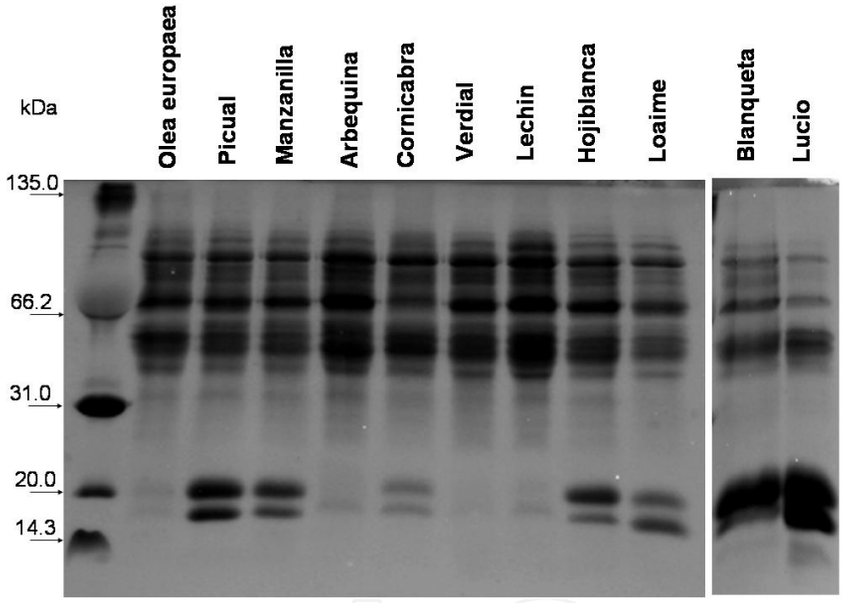

Figure 1. Coomassie stained SDS-PAGE gel of the univarietal pollen extracts and the commercial extract (*Olea europaea*) after using denaturing, reducing conditions. Gels contained 30 μg total protein per lane.

3.2. Immunoblot detection and quantitation of Ole e 1

Immunoblots probed with the monoclonal antibody to Ole e 1 resulted in the presence of two major immunoreactive bands of 18 and 20 kDa, corresponding to the monomeric non-glycosylated and mono-glycosylated forms (Figure 2). Other immunoreactive bands with low quantitative relevance were observed (36, 40 y 44 kDa) in several lanes.

Bands corresponding to the Mw of 18 and 20 kDa were quantitated according to the methods described above. Absolute measurements of the intensity of each individual band and both bands simultanously are displayed in Table 2, as well as the relative percentages calculated as described above.

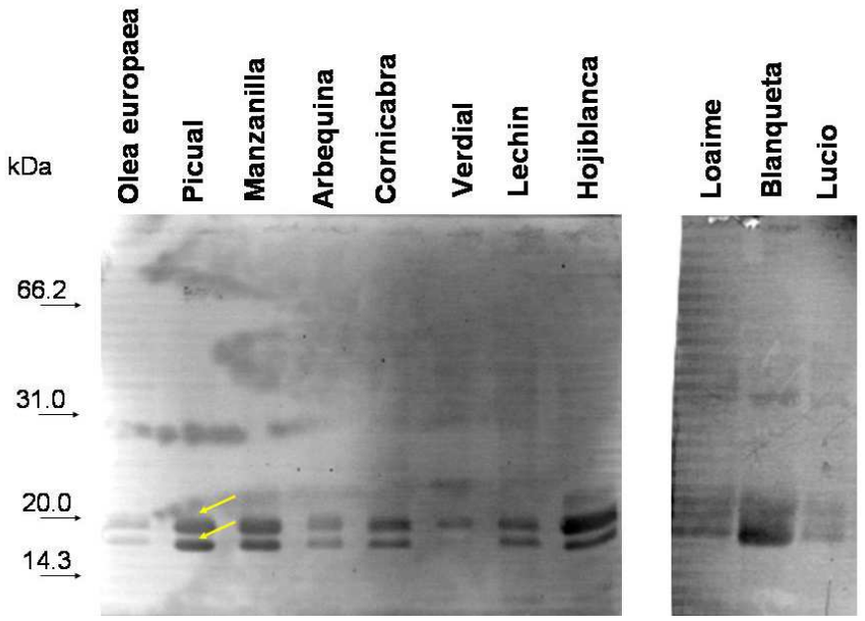

Figure 2. Immunoblot probed with the anti-Ole e 1 monoclonal antibody. Two major bands were observed (yellow arrows), corresponding to apparent molecular weights of 18 and 20 kDa.

	O. europaea	*Picual*	*Manzanilla*	*Arbequina*	*Cornicabra*	*Verdial*	*Lechín*	*Hojiblanca*	*Loaime*	*Blanqueta*	*Lucio*
18 kDa	3672	14385	16746	11894	20033	12174	17668	30706	41345	40388	17436
20 kDa	2348	10666	11233	7983	11716	3204	11804	22780	21143	32768	13087
Σ 18 and 20 kDa	6021	25051	27979	19877	31749	15378	29472	52784	62489	73156	30523
Relative %	8.23	34.24	38.25	27.17	43.40	21.02	40.28	72.15	85.41	100	41.72
18 and 20 kDa	6659	26741	29626	22044	34260	20950	32493	54095	60906	73900	30345
Relative %	9.01	36.18	40.09	29.83	46.36	28.35	43.97	73.21	82.42	100	41.06
Average relative %	8.62	35.21	39.17	28.5	41.88	24.68	42.125	72.68	83.915	100	41.39

Table 2. Quantitation of the two major bands cross-reactive to the anti Ole e 1 antibody. Absolute data in volume units (INT*mm²).

3.3. Immunoblot detection and quantitation of Ole e 2

Immunoblots probed with the polyclonal antiserum to Ole e 2 resulted in the presence of up to five major immunoreactive bands of c.a. 14, 13.7, 14.2, 14.9 and 15.7 kDa (Figure 3).

Bands corresponding to the five Mws were quantitated according to the methods described above. Absolute measurements of the intensity of each individual band and all five bands simultanously are displayed in Table 3, as well as the relative percentages calculated as described above.

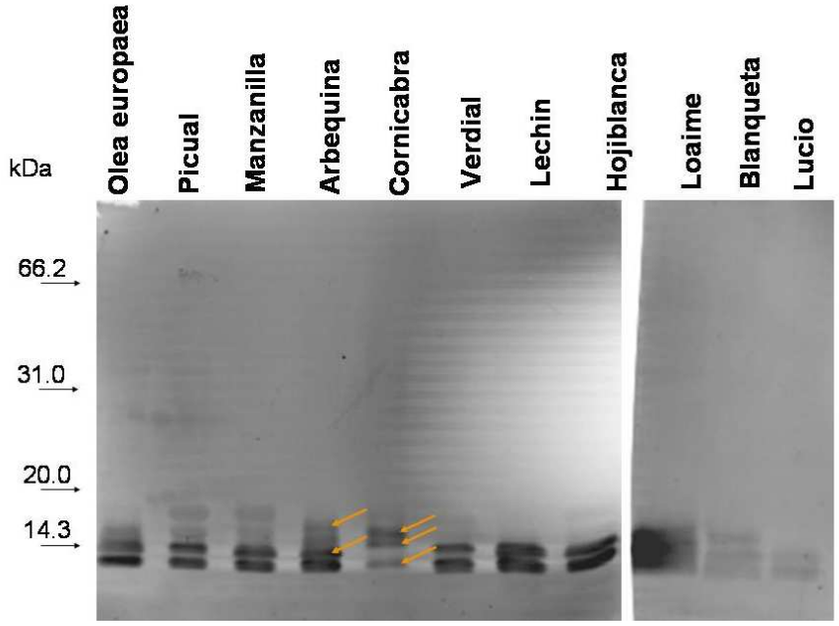

Figure 3. Immunoblot probed with the anti-Ole e 2 polyclonal antiserum. Five major bands were observed (orange arrows), corresponding to apparent molecular weights of 14, 13.7, 14.2, 14.9 and 15.7 kDa.

	O. europaea	*Picual*	*Manzanilla*	*Arbequina*	*Cornicabra*	*Verdial*	*Lechín*	*Hojiblanca*	*Loaime*	*Blanqueta*	*Lucio*
13.0 kDa	357152	277747	248153	405554	165942	394519	537187	356024	910640	476889	350778
13.7 kDa	398025	305569	261364	312528	198300*	250846	484703	463371	1004748	454397	486318
14.2 kDa	264210	198300*	198300*	223593	147305	198300*	198300*	198300*	987772	410790	198300*
14.9 kDa	198300*	198300*	198300*	198300*	290636	198300*	198300*	198300*	198300*	198300*	198300*
15.7 kDa	198300*	198300*	198300*	217632	198300*	198300*	198300*	198300*	198300*	198300*	198300*
Σ bands above	1415987	1178216	1104417	1357607	1000483	1240265	1616790	1414295	3299760	1738676	1431996
Relative %	42.91	35.70	33.47	41.14	30.31	37.59	49	42.86	100	52.70	43.39
All bands	2281004	2385713	2464789	2550651	2499993	2472138	2437099	2312637	2913894	2092950	1973168
Relative %	78.3	81.87	84.59	87.53	85.80	84.84	83.64	79.37	100	71.83	67.71
Average relative %	60.605	58.78	59.03	64.335	58.055	61.215	66.32	61.115	100	62.265	55.55

Table 3. Quantitation of the five major bands cross-reactive to the anti Ole e 2 antibody. Absolute data in volume units (INT*mm^2). *: band not present. The indicated value corresponds to the average of 5 measurements made in the background.

3.4. Immunoblot detection and quantitation of Ole e 5 and Ole e 9

Immunoblots probed with the commercial antibody to Cu,Zn SOD (Ole e 5) and the polyclonal antiserum to Ole e 9 resulted in the presence of up to five major immunoreactive bands of c.a. 16, 16.5, 22, 26 and 50 kDa for Ole e 5, and two immunoreactive bands of c.a. 36 and 46.5 kDa for Ole e 9 (Figure 4).

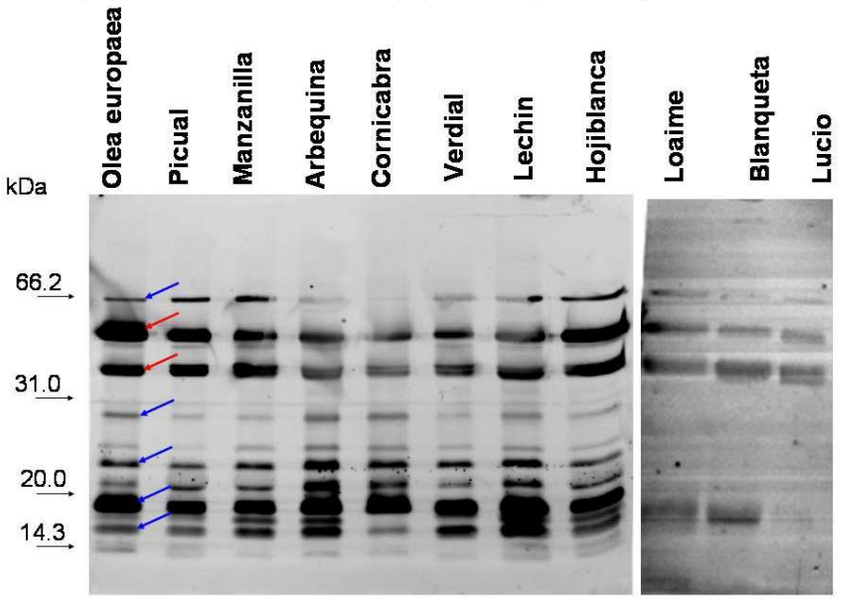

Figure 4. Immunoblot probed with the anti- Cu,Zn-SOD (Ole e 5) commercial antibody and the polyclonal antiserum to Ole e 9. Five major bands were observed (blue arrows), corresponding to apparent molecular weights of 16, 16.5, 22, 26 and 50 kDa for Ole e 5, and two immunoreactive bands of c.a. 36 and 46.5 kDa for Ole e 9 (red arrows).

Bands corresponding to the five Mws of Ole e 5 and two of Ole e 9 were quantitated according to the methods described above. Absolute measurements of the intensity of each individual band and all five bands simultanously are displayed in Tables 4 and 5, as well as the relative percentages calculated as described above.

4. Clustering of cultivars according to their relative allergenic content

Table 6 summarizes the final relative averages of reactivity calculated for each cultivar and allergen. Relative values present a wide range in the case of allergens Ole e 1 and Ole e 9, whereas Ole e 2 and Ole e 5 allergens maintain values relatively constant, higher than 50% for all cultivars, with a single exception (Ole e 5 in the cultivar 'Lucio').

Therefore, the following thresholds have been defined in order to divide cultivars into cultivars with high/average/low allergenic content for the allergens Ole e 1 and Ole e 9. In the case of Ole e 1, we have considered that percentages of 30% and 35% may represent reasonable limits, taking into account the extremely high content of some cultivars in this allergen, which may represents up to 23% of the total protein content for these cultivars (Castro et al. 2003). For Ole e 9, the percentages of 40% and 60% were selected as the thresholds.

	O. europaea	*Picual*	*Manzanilla*	*Arbequina*	*Cornicabra*	*Verdial*	*Lechín*	*Hojiblanca*	*Loaime*	*Blanqueta*	*Lucio*
16.0 kDa	12522	10472	13810	16174	8515	16875	20320	19682	1054	793	714
16.5 kDa	33503	21491	23191	27710	33630	28904	36422	39682	27218	23811	4304
22.0 kDa	9451	7946	9623	14385	12453	9703	13812	17211	6380	5201	5041
26.0 kDa	6221	3389	3688	7134	7617	4447	7775	11424	12698	9191	7098
50.0 kDa	5611	7474	9643	4818	3941	5794	8238	19507	12979	5625	4200
Σ bands above	67308	50772	59955	70221	66156	65723	86567	107506	47350	44621	21357
Relative %	62.61	47.23	55.77	65.32	61.54	61.13	80.52	100	44.04	41.51	19.87
All bands	122909	95529	107694	136847	128879	118503	157103	155113	136354	107779	79135
Relative %	78.23	60.81	68.55	87.11	82.03	75.43	100	98.73	86.79	68.60	50.37
Average relative %	70.42	54.02	62.16	76.215	71.785	68.28	90.26	99.365	65.415	55.055	35.12
Average relative to 100 %	70.87	54.36	62.55	76.70	72.24	68.72	90.83	100	65.83	55.41	**35.34**

Table 4. Quantitation of the five major bands cross-reactive to the anti Cu,Zn SOD (Ole e 5) antibody. Absolute data in volume units (INT*mm²). In this case, the average relative percentage was again referred to 100%, as the maximun relative percentages previously calculated corresponded to two different cultivars ('Hojiblanca' and 'Lechín').

	O. europaea	*Picual*	*Manzanilla*	*Arbequina*	*Cornicabra*	*Verdial*	*Lechín*	*Hojiblanca*	*Loaime*	*Blanqueta*	*Lucio*
36 kDa	20241	18902	25868	18912	15583	17605	24746	44273	29766	28377	20736
46.5 kDa	39567	23419	17751	16764	13584	13916	20517	53338	22784	13160	12730
Σ 36 and 46.5 kDa	59808	42322	43619	35677	29167	31521	45264	97612	52550	41538	33467
Relative %	61.27	43.36	44.68	36.55	29.88	32.29	46.37	100	53.83	42.55	34.28
36 and 46.5 kDa	72676	51950	56389	47169	39589	45075	59672	102195	74531	52213	39717
Relative %	71.11	50.83	55.17	46.15	38.73	44.10	58.39	100	72.93	51.09	38.86
Average relative %	66.19	47.095	49.925	41.35	34.305	38.195	52.38	100	63.38	46.82	36.57

Table 5. Quantitation of the two major bands cross-reactive to the anti Ole e 9 antibody. Absolute data in volume units (INT*mm²).

	O. europaea	*Picual*	*Manzanilla*	*Arbequina*	*Cornicabra*	*Verdial*	*Lechín*	*Hojiblanca*	*Loaime*	*Blanqueta*	*Lucio*	*Thresholds low/average/high*
Ole e 1	8.62	35.21	39.17	28.5	41.88	24.68	42.125	72.68	83.915	100	41.39	30%-35%
Ole e 2	60.605	58.78	59.03	64.335	58.055	61.215	66.32	61.115	100	62.265	55.55	-
Ole e 5	70.87	54.36	62.55	76.70	72.24	68.72	90.83	100	65.83	55.41	35.34	-
Ole e 9	66.19	47.095	49.925	41.35	34.305	38.195	52.38	100	63.38	46.82	36.57	40%-60%

Table 6. Abstract of the relative percentages of reactivity corresponding to the cultivars analysed for each allergen. High reactivity is marked by using bold text, and low reactivity is marked by italics, after considering the thresholds indicated.

The following categories were established after following the above mentioned criteria:

- 'Hojiblanca'-type extract, characterized by high contents of Ole e 1 and Ole e 9. The cultivar 'Loaime' could be included in this same group.
- 'Picual'- type extract, characterized by high content of Ole e 1 and low to average contents of Ole e 9. The cultivars 'Manzanilla', 'Lucio', 'Cornicabra', 'Lechín' and 'Blanqueta' could be included in this same group.
- 'Arbequina' -type extract, characterized by low content in both Ole e 1 and Ole e 9, with average to high contents of Ole e 5 and Ole e 2. The cultivar 'Verdial' could be included in this same group.

The *Olea europaea* commercial extract doesn't match any of the tested cultivars, corresponding to an extract with a relative high proportion of Ole e 9 and low proportion of Ole e 1.

This initial proposal should be implemented by further analyzing additional olive pollen allergens (some of them highly relevant from a clinical point of view like Ole e 7), and by analyzing the allergen profiles of other agronomically relevant cultivars. However, the classification obtained here is in good agreement with the genetic relationships among cultivars already described on the basis of Ole e 1 and Ole e 2 polymorphism (Hamman-Khalifa et al. 2008; Jiménez-López et al., 2012). Moreover, this classification also supports clinical findings describing sharp differences in patient's reactivity to commercially available extracts depending on their place of residence in Spain, where these model cultivars are differentially predominant (Casanovas et al. 1997). Providing that sensitization to specific allergens can be determined in individual patients, the application of the concept of allergenic profile to allergen extracts could be considered a major adventage. This concept would therefore open the posibility of choosing the allergen extract matching the sensitivity of each patient. Moreover, the continuous development of new molecular tools (e.g. new

antibodies with higher specificity) will undoubtedly improve the present type of studies, which has to be considered still preliminary.

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